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ORIGINAL ARTICLE

HCV eradication in recurrent hepatitis C after liver transplantation normalizes enhanced endothelial activation

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SUMMARY

The increased risk of cardiovascular disease (CVD) conferred by hepatitis C virus (HCV) is especially relevant after liver transplantation (LT), but its mechanism is still not well defined. This study aimed to evaluate the influence of HCV eradication in inflammatory and endothelial activation markers after LT. We evaluated inflammatory (TNF-alfa, IL-6, IL-8, and MCP-1) and endothelial activation (E-selectin, ICAM-1, VCAM-1, and MMP-9) markers before and after eradication in 45 LT recipients with HCV infection (LT+/HCV+) and 44 non-transplanted HCV-infected patients (LT-/HCV+). We also considered an additional group of 40 LT recipients without HCV infection (LT+/HCV-). LT+/HCV+ patients presented a higher endothelial activation status before eradication compared with LT+/HCV- patients. However, levels of E-selectin, ICAM-1, VCAM-1, and MMP-9 were comparable between LT+/HCV+ and LT-/HCV+ patients before eradication. HCV eradication decreased ICAM-1 (5466.55 pg/ml vs. 3354.88 pg/ml, $P < 0.001$) and VCAM-1 (10456.52 pg/ml vs. 6658.85 pg/ml, $P < 0.001$) levels in LT+/HCV+ and LT-/HCV+ patients. Remarkably, HCV eradication restored levels of endothelial activation markers of LT+/HCV+ patients compared with that of LT+/HCV- patients. HCV plays a major role in endothelial dysfunction after LT. Furthermore, HCV eradication restores endothelial activation despite the exposure to immunosuppressive therapy.

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Introduction

Hepatitis C virus (HCV) infection is one of the leading causes of morbidity and mortality worldwide with an estimated number of 350,000 deaths each year [1]. In addition to liver damage, chronic HCV infection causes many other relevant extrahepatic complications, especially metabolic disorders and cardiovascular disease (CVD) [2]. Chronic HCV infection has been associated with an increased risk of carotid [3] and coronary [4]

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atherosclerosis, peripheral artery disease [5], cerebral and cardiovascular events [6,7], and cardiovascular mortality [8]. Furthermore, it increases the prevalence of metabolic disorders in patients infected with HCV compared with non-infected subjects, specifically in terms of diabetes mellitus (DM) [9] and insulin resistance (IR) [10].

Not surprisingly, recurrence of HCV after liver transplantation (LT) has been also associated with the development of IR, DM [11], and metabolic syndrome. Likewise, metabolic syndrome is highly prevalent in LT recipients, affecting approximately half of this patient population [12], thus increasing the risk of cardiovascular events after LT [13]. Moreover, CVD is a major problem in LT recipients leading to an increase in morbidity and in short- and long-term mortality [14]. Furthermore, immunosuppressive therapy can be a contributing factor in developing cardiovascular risk (CVR) after LT [15]. Therefore, the increased risk of CVD conferred by chronic HCV infection may be especially relevant in the post-transplant setting.

Chronic inflammatory cytokines production caused by HCV infection contributes to developing CVD due to various effector mechanisms, including increase in intracellular adhesion molecules, the generation of oxidative stress and IR [16]. Increased levels of intracellular adhesion molecules have been associated with atherosclerotic disease and an adverse cardiovascular prognosis [17,18]. Additionally, HCV infection in patients coinfected with HCV and the human immunodeficiency virus (HIV) has been related to the development of endothelial dysfunction that increases intracellular adhesion molecules levels [19]. Prospective studies conducted in HCV/HIV coinfected patients treated with new direct antiviral agents (DAA) [20] have shown a sustained and significant decrease in intercellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) after terminating antiviral treatment in the subgroup of patients with sustained virologic response (SVR) compared with baseline levels. In addition to the impact of HCV eradication on the levels of endothelial activation biomarkers, a reduction in the incidence of atherosclerosis and cardiovascular events has been described [21,22], indicating that ameliorating endothelial activation after HCV eradication is accompanied by improved CVD. However, whether this beneficial effect is similar in patients after LT in which other CVR factors have been characteristically implicated is largely unknown. Thus, our study aimed to evaluate the influence of HCV eradication in inflammatory and endothelial activation markers and IR, in recurrent HCV infection after LT.

Patients and methods

Patients

Forty-five consecutive LT recipients were prospectively recruited at the Hospital General Universitario Gregorio Marañón, Madrid, Spain. Patients were included if 1) they had HCV recurrence after LT, based on the detection of HCV-RNA in serum by reverse transcription–polymerase chain reaction (RT-PCR) and histological confirmation; and 2) they underwent IFN-free, DAA-based therapy (LT+/HCV+ group). Exclusion criteria were as follows: 1) baseline liver disease or graft dysfunction of etiology different of HCV or due to mixed etiology (i.e., excessive alcohol consumption, hepatitis B, recurrence of autoimmune liver disease, acute or chronic rejection); 2) HIV infection; and 3) presence of acute processes that could modify the inflammatory patient´s condition (i.e., infections, neoplasia, acute or chronic rejection, and rheumatic disease).

To assess the potential restoration of endothelial activation after HCV eradication, we considered two different control groups. Firstly, a group of non-transplanted HCV-infected patients who underwent IFN-free, DAA-based therapy at the Hospital General Universitario Gregorio Marañón within the same time frame matched in a 1:1 ratio with transplanted patients for age (± 5 years), sex, and severity of liver fibrosis estimated with transient elastography (TE) (± 2 kPa) (LT-/HCV+ group). Secondly, we evaluated an additional control group of LT recipients without HCV infection followed up at the Hospital General Universitario Gregorio Marañón within the same time frame, matched in a 1:1 ratio with LT+/HCV+ patients for age (± 5 years), sex, and transplant date (± 1 year) (LT+/HCV- group).

All patients gave their written informed consent, and the study was performed according to the International Ethical Guidelines for Epidemiological Studies (Council for the International Organizations of Medical Sciences, Geneva, Switzerland, 2008) and the Declaration of Helsinki (Seoul, South Korea, 2008).

Clinical and laboratory assessment

In HCV-treated patients, clinical and laboratory data and blood samples were collected before starting DAA therapy and 12 and 72 weeks after the end of treatment. In the LT+/HCV- group, clinical and laboratory data

were collected in a comparable time frame after LT. Pre- and post-DAA treatment (72 weeks after the end of antiviral therapy) liver stiffness was assessed by TE using a FibroScan® 502, and the results were expressed in Kilopascals (KPa). Blood samples were collected after an 8-hour overnight fasting period for measuring study parameters (see below). Serum aliquots obtained were stored at −80°C until its processing.

CVD was defined as coronary artery disease (clinically diagnosed with stable or unstable angina, or angiographic findings of atherosclerotic stenosis >50% in 1 or more of the 3 major coronary arteries or myocardial infarction), congestive heart failure, stroke or transient ischemic attack, arrhythmias, cardiac arrest, and symptomatic peripheral vascular disease [23]. The DM diagnosis was defined per the American Diabetes Association guidelines [24]. Arterial hypertension was defined as having systolic pressure >140 mm Hg, diastolic pressure >90 mm Hg or when the patient was receiving treatment with drugs or diet. Dyslipidemia was defined as requiring lipid-lowering medication or based on the following laboratory findings: triglycerides ≥150 mg/ml or total cholesterol >200 mg/dl with LDL >130 mg/dl.

The IR degree was estimated by the homeostatic model assessment method (HOMA) described by Matthews [25] in patients who did not receive any antidiabetic treatment. The HOMA model is used to estimate insulin sensitivity and beta cell function from fasting plasma insulin and glucose concentrations. The interrelation between glucose and insulin reflects the balance between hepatic glucose output and insulin secretion [26]. Cumulative tacrolimus exposure was assessed by the area under curve of trough concentrations (AUCtc) using the Wagner–Nelson equation. Tacrolimus trough levels were determined within 12 months before starting DAA, during therapy, and 18 months after treatment completion.

Serum biomarkers evaluation

Frozen serum aliquots were defrosted and several biomarkers' serum levels were measured using the commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D Systems Europe Ltd., UK). To evaluate the degree of systemic inflammation, we determined serum levels of tumor necrosis factor alfa (TNF-alfa), interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein 1 (MCP-1). Additionally, to assess endothelial activation we measured soluble E-selectin, soluble ICAM-1, soluble VCAM-1, and matrix metallopeptidase 9 (MMP-9).

Antiviral therapy

All patients received DAA-based antiviral therapy according to the EASL guidelines available at the time of enrollment [27]. All patients were tested at baseline for HCV-RNA with RT-PCR and HCV genotype. HCV-RNA was determined 12 weeks after treatment completion. SVR was defined as undetectable HCV-RNA 12 weeks after the end of antiviral therapy.

Statistics

Quantitative variables are expressed as mean (SD) and qualitative variables as n (%). Categorical data were analyzed using the chi-squared test or the Fisher's exact test. Quantitative variables were compared using t-Student or Wilcoxon and Mann–Whitney U tests, as appropriate. Changes in laboratory determinations at different time points were assessed by one-way ANOVA followed by Scheffe's test for multiple mean comparisons when a significant main effect was found. Pearson´s correlation was used to evaluate the association between quantitative variables. A two-tailed P-value <0.05 was considered statistically significant. Statistical analysis was performed by STATA Version 13.0.

Results

Patients' characteristics and prevalence of CVR factors

A total of 135 patients were initially included, with 45 patients in each of the three study groups. One out of the 45 LT-/HCV+ patients and three out of the 45 LT+/HCV- recipients were lost in follow-up and were thus excluded from the study; two additional patients belonging to the LT+/HCV- patients group were excluded due to withdrawal of consent. Therefore, the final study population comprised 45 LT+/HCV+, 44 LT-/HCV+, and 40 LT+/HCV- patients. The characteristics of HCV-infected patients, with LT (LT+/HCV+) and without LT (LT-/HCV+), are shown in Table 1. The vast majority of patients presented compensated cirrhosis and mild hepatic impairment (Child–Pugh class A). Among traditional CVR factors, LT+/HCV+ patients had a significantly higher prevalence of DM, arterial hypertension, dyslipidemia, and chronic kidney disease as compared to the LT-/HCV+ patients. Eight out of 45 LT+/HCV+ patients (17.78%) and 7 out of 44 LT-/HCV+ (15.91%) ($P = 0.52$) presented CVD before HCV

therapy. During the study period, 3 patients in the LT+/HCV+ group and 2 in the LT-/HCV+ group developed new onset of CVD. No relevant changes were observed regarding the prevalence of CVR factors before and after HCV treatment in the LT+/HCV+ and LT-/HCV+ group (Table 1).

Data related to the characteristics of HCV infection and DAA therapy are shown in Tables S2 and S3. All patients reached SVR.

The LT recipients' characteristics, with (LT+/HCV+) and without HCV infection (LT+/HCV-), are shown in Table 1. The majority of patients received tacrolimus as the immunosuppressive treatment. LT+/HCV- recipients received mycophenolate mofetil in a significantly greater proportion compared with LT recipients with HCV infection.

Comparison of inflammatory and endothelial activation biomarkers' profile between LT+/HCV+ and LT-/HCV+ patients

To assess the effect of LT and the potential impact of immunosuppressive therapies on the mechanisms of CVD in HCV patients, we compared the inflammatory and endothelial activation biomarkers' profile in LT+/HCV+ and LT-/HCV+ patients (Table 2). LT+/HCV+ patients showed similar levels of TNF-alfa, IL-6, IL-8, MCP-1, soluble E-selectin, soluble VCAM-1, soluble ICAM-1, and MMP-9 before HCV eradication compared with the LT-/HCV+ group. HOMA-IR (3.39 [2.57] vs. 10.53 [19.82], $P = 0.166$). However, LT+/HCV+ patients had a significantly greater DM prevalence (21/45 [46.67%] vs. 8/44 [18.8%]; $P = 0.004$).

Table 1. Comparison of baseline characteristics according to the study group (with and without LT).

	LT+/HCV+ ($n = 45$)	LT-/HCV+ ($n = 44$)	P*	LT+/HCV- ($n = 44$)	$P^{\dagger}$
General characteristics					
Age (years)	61.87 (8.34)	60.68 (7.47)	0.482	59.98 (8.38)	0.301
Sex (male)	34 (75.56)	33 (75)	0.952	29 (72.50)	0.807
Cardiovascular disease	8 (17.78)	7 (15.91)	0.814	10 (25.00)	0.439
Diabetes mellitus	21 (46.67)	8 (18.18)	0.004	14 (35.00)	0.377
Arterial Hypertension	33 (73.33)	15 (34.09)	0.000	22 (55.00)	0.111
Dyslipidemia	13 (28.89)	4 (9.09)	0.018	16 (40.00)	0.360
Chronic kidney disease	13 (28.89)	1 (2.27)	0.001	13 (32.50)	0.815
History of active smoking	7 (15.56)	11 (25)	0.244	2 (5.00)	0.114
Interval since LT (years)	8.37 (6.12)	NA	NA	8.22 (6.25) 0.910	
Liver disease					
Baseline liver stiffness (by TE)	13.42 (8.04)	14.29 (9.22)	0.641	NA	NA
Baseline MELD	8.5 (3.45)	6.69 (1.24)	0.004	NA	NA
Decompensated disease	1 (2.22)	0 (0)	0.326	NA	NA
Child–Pugh (A/B/C)	44 (97.78)/1 (2.22)/0 (0)	41 (97.62)/1 (2.38)/0 (0)	1.000	NA	NA
Laboratory data					
Bilirubin (mg/dl)	1.03 (0.55)	0.68 (0.31)	0.000	0.67 (0.41)	0.001
INR	1.16 (0.51)	1.11 (0.30)	0.608	1.13 (0.31)	0.784
Albumin (g/dl)	4.02 (0.54)	4.31 (0.31)	0.003	5.58 (7.18)	0.170
AP (U/l)	109.24 (39.09)	81.25 (28.95)	0.000	90.45 (34.58)	0.022
ALT (U/l)	60.38 (39.09)	76.59 (58.81)	0.128	21.53 (13.60)	0.000
Creatinine (mg/dL)	1.06 (0.26)	0.85 (0.17)	0.000	1.07 (0.27)	0.882
HOMA-IR	3.39 (2.57)	10.53 (19.82)	0.166	5.79 (16.67)	0.583
Immunosuppression					
Tacrolimus	29 (64.44)	NA	NA	25 (62.50)	1.000
MMF	8 (17.78)	NA	NA	19 (47.50)	0.005
mTOR inhibitors	11 (24.44)	NA	NA	5 (12.50)	0.178
Ciclosporin	7 (15.56)	NA	NA	6 (15.00)	1.000

Data are expressed as mean (SD) or n (%).

TE, transient elastography; HOMA, homeostatic model assessment.

*Comparison between LT+/HCV+ and LT-/HCV+ patients.

†Comparison between LT+/HCV+ and LT+/HCV- patients.

Table 2. Comparison of baseline inflammatory and endothelial activation biomarkers of HCV-infected patients according to the study group.

	LT+/HCV+	LT-/HCV+	*P*
TNF-alfa (pg/ml)	10.20 (5.36)	6.97 (2.90)	0.501
IL-6 (pg/ml)	3.97 (3.96)	4.02 (4.32)	1.000
IL-8 (pg/ml)	45.88 (43.18)	35.46 (30.82)	0.966
MCP-1 (pg/ml)	317.40 (176.21)	295.27 (135.23)	0.875
sE-selectin (pg/ml)	462.73 (297.71)	507.24 (282.45)	0.790
sICAM-1 (pg/ml)	6004.83 (2173.82)	6236.63 (3566.35)	0.941
sVCAM-1(pg/ml)	14370.55 (9259.37)	10850.09 (9586.89)	0.224
MMP-9 (pg/ml)	1771.21 (1578.45)	2174.28 (1397.39)	0.768

Data are expressed as mean (SD).

To evaluate whether HCV eradication could restore the endothelial activation profile, we assessed the related biomarkers after the end of antiviral treatment. Remarkably, HCV eradication improved endothelial activation both in LT+/HCV+ and in LT-/HCV+ patients. Thus, a statistically significant reduction of soluble ICAM-1 and soluble VCAM-1 levels and an increase of MMP-9 levels were observed after 72 weeks after terminating antiviral treatment (Figure 1). However, no changes were observed on inflammatory biomarkers after HCV eradication (TNF-alfa, IL-6, IL-8, and MCP-1) (Table S4). Additionally, a statistically significant reduction of soluble E-selectin levels was observed in LT-/HCV+ patients. The magnitude of the observed changes in the endothelial activation markers was similar in both groups at 12 weeks (Figure 1). Moreover, regardless of the LT recipients being exposed to immunosuppression, the improvement on endothelial activation was similar in both groups at 72 weeks after ending treatment (Figure 1). Only MMP-9 showed a significantly greater increase in LT+/HCV+ patients at 72 weeks (Figure 1).

Insulin resistance estimated by HOMA-IR improved early after HCV eradication in in LT-/HCV+ patients (10.53 [19.82] vs. 2.80 [1.67], $P = 0.035$), but not in LT+/HCV+ recipients (3.68 [2.57] vs. 2.69 [2.06], $P = 0.197$) (Figure 2). Similar results were observed in HOMA-IR at 72 weeks after ending antiviral treatment in both groups (LT-/HCV+: 11.09 [20.65] vs. 3.37 [2.50], $P = 0.038$; LT+/HCV+: 3.39 [2.60] vs. 3.58 [2.65], $P = 0.828$).

Additionally, baseline levels of TNF-alfa and IL-6 were tightly correlated with soluble ICAM-1 and soluble VCAM-1 levels (Figure S1). There was no correlation observed between other serum biomarkers. The degree of liver fibrosis estimated by elastography showed a weak, although statistically significant, correlation with soluble ICAM-1 ($r = 0.26$, $P = 0.046$) and soluble VCAM-1 ($r = 0.33$, $P = 0.01$) mean levels before HCV eradication.

Additional analysis comparing levels of inflammatory and endothelial activation markers and HOMA-IR before and after treatment according to the HCV genotype was performed. No significant differences were observed (data not shown).

Comparison of the inflammatory and endothelial activation biomarkers' profile between LT+/HCV+ and LT+/HCV- patients

To assess the HCV infection's effect on the mechanisms of CVD after LT, we evaluated the inflammatory and endothelial activation biomarkers' profile in LT recipients with and without HCV infection. LT+/HCV+ recipients presented a higher endothelial activation status before eradication compared with non-infected LT recipients. Baseline mean levels of soluble E-selectin, soluble ICAM-1, and soluble VCAM-1 were significantly greater in LT+/HCV+. Furthermore, significantly lower mean levels of MMP-9 were observed in LT+/HCV+ group (Figure 3). However, both groups had a similar degree of IR estimated by HOMA-IR before virus eradication (3.39 [2.60] vs. 5.79 [16.67], $P = 0.583$).

Seventy-two weeks after ending antiviral treatment, LT+/HCV+ and LT+/HCV- recipients had similar serum levels of E-selectin, ICAM-1, VCAM-1, and MMP-9 (Figure 3), indicating that HCV eradication restored the endothelial activation status of LT+/HCV+ recipients. However, HCV eradication did not modify the severity of IR at the medium term in LT +/HCV+ recipients (3.58 [2.65] vs. 5.79 [16.67], $P = 0.550$).

Finally, cumulative exposure to tacrolimus was similar in LT+/HCV+ and LT+/HCV- groups (AUCtc 3960.24

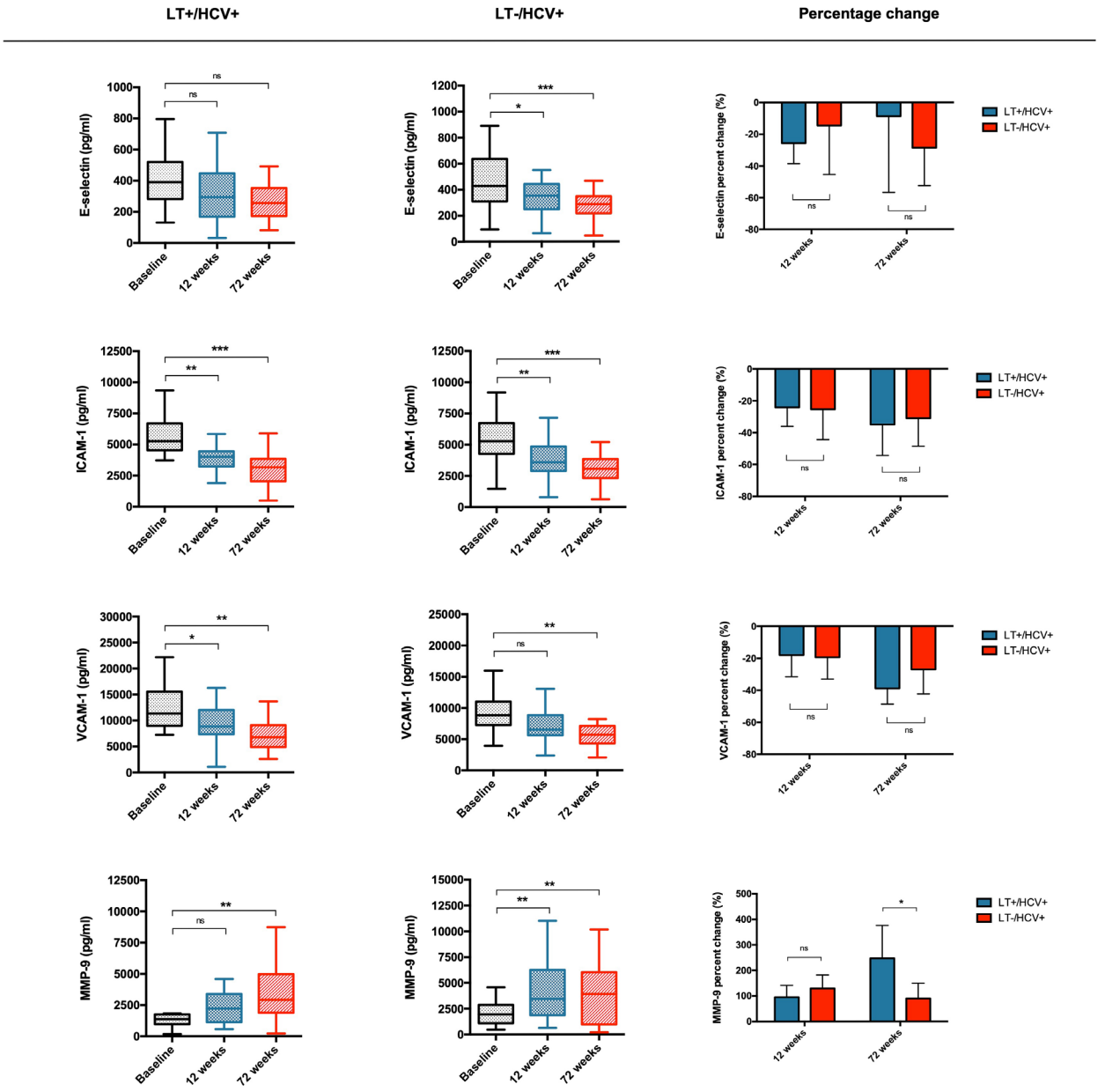

Figure 1 Changes in endothelial activation markers associated with HCV eradication in LT+/HCV+ patients and LT-/HCV+ patients (12 and 72 weeks). Comparison of percentage change of endothelial activation markers between both groups. The box ranges from Q1 (the first quartile) to Q3 (the third quartile) of the distribution and the range represents the IQR (interquartile range). The median is indicated by a line across the box. The "whiskers" on box plots extend from Q1 and Q3 to the most extreme data points.

[1563.33] vs. 3675.32 [2041.90], $P = 0.53$). Moreover, the intensity of tacrolimus exposure was not associated with endothelial activation biomarkers. In fact, we did not find differences in serum levels of inflammatory and endothelial activation markers between patients with low cumulative exposure to tacrolimus (AUCtc Quartile 1) and those with greater cumulative exposure to tacrolimus (AUCtc Quartile>1) in the 3 months previous to laboratory determinations (Table 3).

Discussion

DAA therapy's positive impact in graft function and in the natural history of HCV recurrence after LT has been well described elsewhere [28]. However, besides liver damage, chronic HCV infection has been associated with endothelial dysfunction, atherosclerosis, and CVD [4,6,7,19,29], which is especially important after LT due to its relevant prognostic implications [14]. Thus,

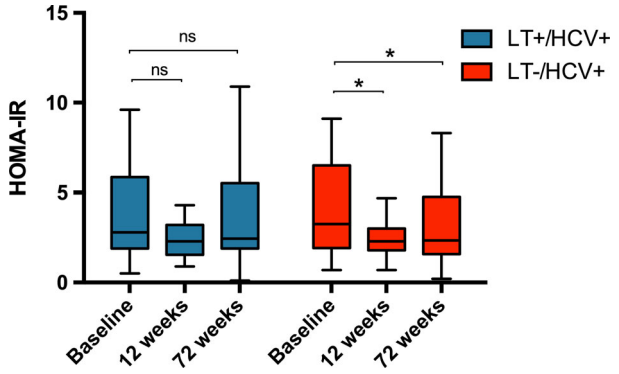

Figure 2 Changes in HOMA-IR associated with HCV eradication in LT+/HCV+ patients and LT-/HCV+ patients (12 weeks). The box ranges from Q1 (the first quartile) to Q3 (the third quartile) of the distribution and the range represents the IQR (interquartile range). The median is indicated by a line across the box. The "whiskers" on box plots extend from Q1 and Q3 to the most extreme data points.

eradicating HCV could significantly impact the morbidity and mortality derived from CVD after LT through improving endothelial dysfunction.

Our data demonstrate for the first time that HCV eradication with new DAA significantly improves the marked endothelial activation observed after HCV recurrence. Thus, we found a significant reduction of ICAM-1 and VCAM-1 levels after eliminating the virus that were maintained 72 weeks after the end of therapy. This fact has been demonstrated already in non-transplant patients following HCV eradication with IFN-based therapy [30] and DAA [20,31]. Endothelial cell activation produces an increased expression of adhesion molecules that activate essential processes promoting the induction, evolution, and destabilization of atherosclerotic lesion. These molecules' serum levels increase in parallel with CVR factors and have been related to atherosclerotic disease as well as with CVD. Circulating VCAM-1 is increased in patients with acute myocardial infarction and coronary heart disease [32], while levels of soluble ICAM-1 are elevated in coronary circulation in patients with unstable and stable angina or acute myocardial infarction [33,34], and is associated with early stages of atherosclerosis development [35].

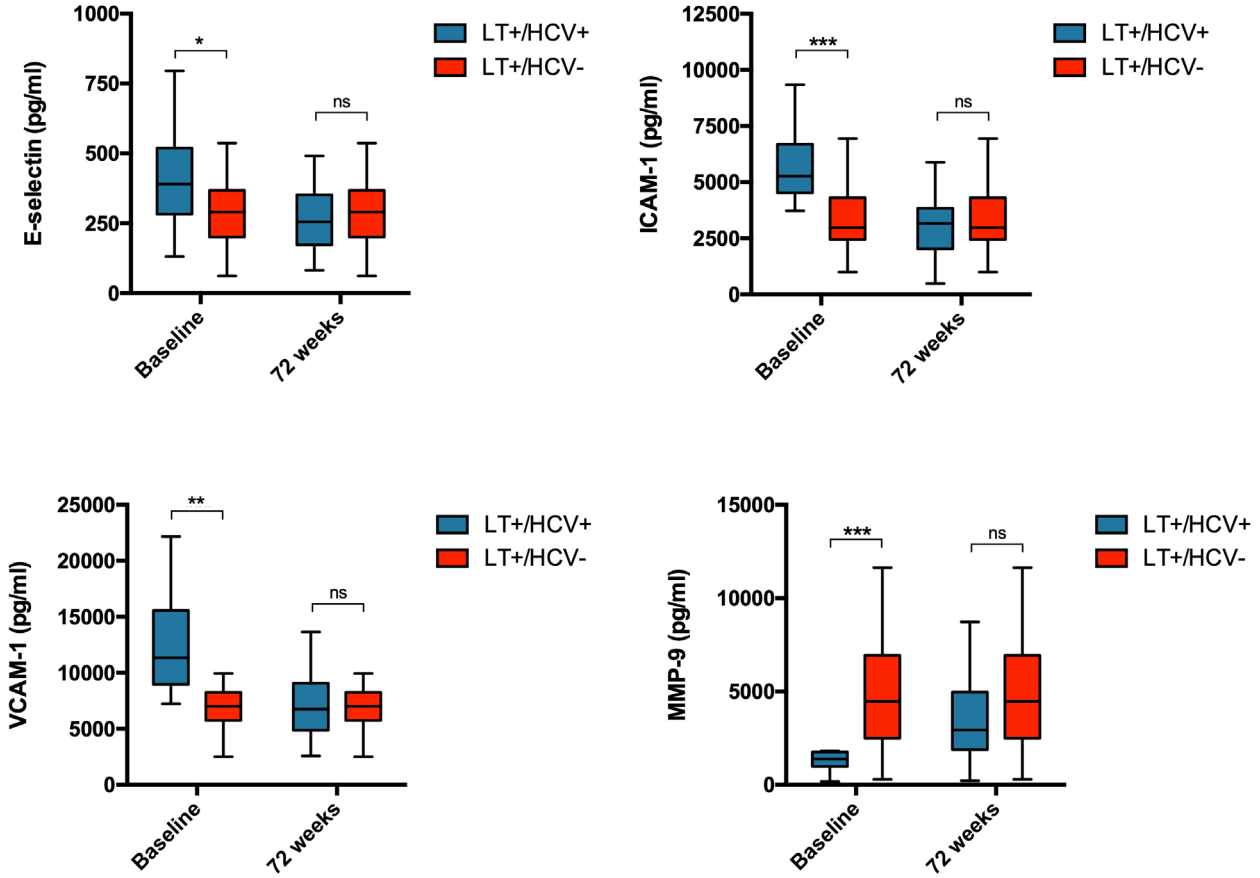

Figure 3 Comparison of baseline and 72-week levels of endothelial activation biomarkers of LT+/HCV+ patients and LT+/HCV- patients. The box ranges from Q1 (the first quartile) to Q3 (the third quartile) of the distribution and the range represents the IQR (interquartile range). The median is indicated by a line across the box. The "whiskers" on box plots extend from Q1 and Q3 to the most extreme data points.

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Table 3. Comparison of serum biomarkers according to area under curve of trough concentrations (AUCtc) of tacrolimus quartile in LT recipients (with and without HCV infection).

LT+ (with and without HCV)			
	AUCtc Q1	AUCtc > Q1	*P*
TNF-alfa (pg/ml)	9.39 (2.53)	10.97 (12.96)	0.712
IL-6 (pg/ml)	4.96 (4.68)	5.97 (16.50)	0.858
IL-8 (pg/ml)	129.67 (148.40)	102.48 (191.76)	0.691
MCP-1 (pg/ml)	328.67 (124.47)	290 (175.99)	0.534
sE-selectin (pg/ml)	234.11 (79.34)	292.62 (110.49)	0.148
sICAM-1 (pg/ml)	3021.78 (1858.93)	3735 (1729.42)	0.291
sVCAM-1 (pg/ml)	6648.56 (3883.34)	7525.68 (2437.76)	0.414
MMP-9 (pg/ml)	6536.11 (4011.56)	4039.84 (2763.49)	0.038

Data are expressed as mean (SD).

Finally, E-selectin serves as a molecular marker for atherosclerosis and the development of coronary heart disease [18,32]. Consequently, cellular adhesion molecules can be considered as biomarkers for early atherosclerosis and preclinical CVD, which gives relevance to our data.

Another relevant finding of our investigation is that serum levels of endothelial activation biomarkers after DAA in LT+/HCV+ were similar to those observed in non-HCV-transplanted patients. This is an outstanding finding considering that baseline serum levels of E-selectin, ICAM-1, and VCAM-1 in LT+/HCV+ patients were significantly greater than in LT patients without HCV infection (LT+/HCV-). Altogether, our results indicate the HCV infection's essential role in generating endothelial dysfunction after LT and its amelioration after HCV eradication.

Infectious agents may contribute to atherosclerotic development by chronic inflammation, either directly through infection of vascular cells or indirectly through systemic inflammatory response [36]. Specifically, HCV infection is associated with atherosclerosis by increasing local and systemic inflammation [37–39]. Thus, HCV induces inflammatory cytokines, consequently increasing intracellular adhesion molecules and endothelial activation. In the current study, there were no significant changes observed on the levels of TNF-alfa, IL-6, IL-8, and MCP-1 after HCV eradication. Furthermore, these inflammatory markers' levels were similar between both groups and, interestingly, also similar to the levels found in healthy individuals in other studies [40]. Our findings suggest that HCV generates endothelial activation through a direct cytopathic effect of the virus on the endothelium, thus generating an increase in concentration of soluble intercellular adhesion molecules and making chronic systemic inflammation a secondary mechanism. HCV genomic sequences have been detected in plaque tissues, suggesting an active infection of the atherosclerotic plaques [41]. Consequently, HCV seems to promote endothelial dysfunction and play a role in atherosclerosis through local action.

Remarkably, DAA's favorable effects on endothelial activation in LT+/HCV+ patients were observed despite the presence of the characteristic CVR factors associated with LT. In fact, we confirmed that LT+/HCV+ patients more frequently presented CVR factors as DM, arterial hypertension, dyslipidemia, and chronic kidney disease compared with LT-/HCV+ patients, suggesting the possibility of a higher degree of endothelial activation. However, both groups showed a similar pre-treatment profile of endothelial activation biomarkers, probably indicating the HCV infection's main role besides other CVR. We must note that adequately controlling CVR factors with medication and lifestyle interventions could explain our results, equating the degree of endothelial activation to that of non-transplanted patients. Furthermore, implementing a specific protocol for managing asymptomatic coronary artery disease in LT candidates in our center [42] may have contributed to selecting a population with a favorable CVR profile, without significant preclinical coronary artery disease, or treated with revascularization if present.

Furthermore, the cumulative exposure to tacrolimus has a non-relevant impact on endothelial activation biomarkers in LT recipients. Patients with low cumulative exposure to tacrolimus showed similar levels of endothelial activation markers to those with higher cumulative exposure to tacrolimus. This is in agreement with some studies suggesting that tacrolimus may have beneficial effects on endothelial function [43].

Specifically, tacrolimus decreases the production of cytokines such as TNF-alfa and IL-6, and reduces the expression of adhesion molecules VCAM-1, ICAM-1, and E-selectin in vitro [44]. Similar effects have been described with proliferation inhibitors such as mycophenolate mofetil [45] and glucocorticoids [46]. Thus, the immunosuppressive drugs may impact CVD in terms of contributing to generate or aggravate CVR factors in LT recipients, and not through a direct action on endothelial activation. Our results suggest that an adequate control of post-transplant CVR factors and the HCV eradication are key factors that impact endothelial activation and consequently the development of CVD.

An intriguing finding of our observation is the interpretation of matrix metalloproteinases levels. Matrix metalloproteinases seem to be involved in the atherosclerotic plaque growth, destabilization, and eventually plaque rupture [47]. In our study, we observed a significant increase of MMP-9 levels in LT recipients with HCV infection and non-transplanted HCV-infected controls after eradicating the virus. Moreover, LT recipients with recurrent HCV showed lower levels of MMP-9 before eradication compared with the transplanted control group, increasing to comparable levels after virus eradication. While several studies suggest detrimental effects of the MMP-9 overexpression on the development of atherosclerosis, controversy exists in this respect. Interestingly, some studies suggest that MMP-9 signaling may inhibit fibrin deposition and platelet aggregation and promote a decrease in thrombus size, suppressing the progression of atherosclerosis [48–51]. Therefore, MMP-9's atherogenic effects remain controversial and there is evidence that supports its protective effect.

Our study's strengths include its novelty regarding the effect of HCV eradication with new DAA regimens on endothelial activation in LT recipients. Additionally, this is the first study to suggest that endothelial activation in LT recipients with HCV recurrence depends essentially on the virus direct effect on the endothelium, being that other circumstances classically associated with the course of LT were less significant than expected. Importantly, eradicating the virus restores the endothelial activation state to the levels observed in LT patients who were never HCV-infected. This could lead to a significant reduction in CVR in the medium–long term through an improvement in endothelial dysfunction. Although in the current study we did not identify a decrease in CVD, probably due to the short follow-up, we postulate that eradicating HCV with new DAA in LT recipients could lead to a significant decrease in long-term non-liver-related mortality and should be evaluated in future studies. Finally, our study highlights the relevance of chronic viral infections in endothelial dysfunction and potential accelerated atherosclerosis after LT. Chronic HCV infection could serve as a model for other chronic viral infections such as cytomegalovirus, which has been associated with an increased risk of cardiovascular events in HCV LT patients [52]. Specific studies should evaluate the impact of chronic viral infections in endothelial activation and preclinical atherosclerosis in LT recipients.

Our study is not without limitations. Besides the relatively small sample size, we could not incorporate preclinical atherosclerosis tests to confirm the correlation between the decrease in endothelial dysfunction markers and the improvement of preclinical disease. However, the association between the biomarkers used in our study and preclinical and clinical CVD has been widely demonstrated in previous studies. Moreover, it could be argued that the group-matching design could have generated a selection bias and reduce the validity of our results. However, the study groups were only matched by age, sex, and degree of liver fibrosis, which is a key factor since it can modify the determined biomarkers' levels. The relatively high proportion of smokers may be considered an additional limitation of our study, since active smoking may potentially impact on circulating levels of endothelial activation markers. However, the proportion of smokers was similar between the study groups. Another limitation is the lack of an additional control group that could have indicated normal biomarkers values. Nonetheless, our data are consistent with previous studies regarding the effect of HCV eradication [31,53] and the biomarkers' values observed in HCV-negative individuals [20]. Finally, it could be considered that the improvement of liver function and, potentially, of portal hypertension/bacterial translocation could be the reason for our results. However, the vast majority of our patients presented compensated cirrhosis and baseline levels of inflammatory markers' were similar to the levels found in healthy individuals [40]. Therefore, the presence of pathological bacterial translocation and systemic inflammation as the main mechanism of endothelial activation seems unlikely.

In conclusion, this study demonstrates that HCV eradication with DAA is associated with a significant reduction of endothelial activation in transplanted and non-transplanted patients. This finding is especially relevant in LT recipients with HCV recurrence in which the beneficial effect of HCV eradication is observed despite

the exposure to immunosuppressive therapy. Since CVD is one of the most common causes of death after the first year of LT, endothelial activation through eradicating HCV may impact long-term survival and should be evaluated in future studies.

Authorship

A.A performed research design, data collection, analysis, and writing of the article. M.R. performed research design, data collection, analysis, and writing of the article. M.P performed research design, data collection, and analysis. A.F. performed research design and data collection. L.D performed research design and data collection. C.N performed research design and data collection. A.C performed research design and data collection. F.D. performed research design and data collection. P.C performed research design and article review. D.R. performed research design and article review. J.L performed research design and article review. R.B performed research design, analysis, and article review. M.S performed research design, analysis, article review, and supervision of the project.

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Conflicts of interest

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Correlation between baseline endothelial activation biomarkers and systemic inflammation biomarkers in LT+/HCV+ patients.

Table S1. Comparison of inflammatory biomarkers of HCV-infected patients according to the study group 12 and 72 weeks after HCV eradication.

Table S2. HCV genotype in HCV infected patients according to the study group (with and without LT).

Table S3. Direct-acting antiviral agents used in HCV infected patients according to the study group (with and without LT).

Table S4. Comparison of inflammatory biomarkers of HCV-infected patients according to the study group 12 and 72 weeks after HCV eradication.

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